REMARKS

Claims 1, 29-32, and 35-43 were examined in this case. Claims 31, 36, and 41 are objected to based on formalities. Claims 1, 29-32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 101. Claims 30 and 35 stand rejected under 35 U.S.C. § 112, second paragraph. Claims 1, 29-32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 112, first paragraph. Claims 1, 29, 31, 32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 102(a). Each of these issues is addressed below.

Claim Objections

Claims 31, 36, and 41 stand objected to based on formalities. These claims have been amended as suggested by the Examiner, and the objection may be withdrawn.

Rejection under 35 U.S.C. § 101

Claims 1, 29-32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 101 on the basis that the claimed invention is directed to non-statutory subject matter. As applied to the currently amended claims, which recite "isolated" proteins, nucleic acids, or cells, this rejection may be withdrawn.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 30 and 35 stand rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to distinctly claim the subject matter.

Claim 30 has been amended as recommended by the Examiner.

Claim 35 has been amended to correct the term "the insertion," as recommended by the Examiner, and to amend the Markush language. The terms "a foreign gene" and "a signal" have been deleted.

The rejections under § 112, second paragraph may be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1, 29-32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement and written description requirements. As applied to the amended claims, these rejections are respectfully traversed.

The present rejections turn on the assertion that Applicants' prior claim language did not adequately define the recited insertion sites and, with respect to claim 35, a "signal" for double-strand synthesis was not provided in the specification. Without agreeing with the Office, Applicants have removed the term "signal for double strand synthesis" from the claim, obviating this second basis for rejection.

With respect to the claim language issue, the Office is directed to Applicants' current claims. As amended, these claims require that an insertion be located directly adjacent to at least one amino acid in the recited sequences. In view of this clarifying amendment, it cannot be asserted that Applicants' claims cover essentially any insertion site. Rather, it is clear that an insertion according to the present claims must be made directly before or directly after an amino acid in one of seven, specifically recited AAV capsid positions.

Moreover, no evidence of record has been presented that would indicate that insertions at these *specifically recited positions* would either be unworkable or their function unpredictable. Indeed, all evidence to date indicates that these AAV capsid sites are amenable to insertions that result in viral re-targeting. As previously made of record, the present specification as well as publications by Grifman, Nicklin, and Wu demonstrate that insertions at SEQ ID NO:7 are well tolerated and result in retargeting (i.e., increasing) cell infectivity. Furthermore, the previously submitted reference from Wu indicates that every site encompassed by Applicants' claims that was tested by Wu was amenable to insertions suitable for retargeting. In particular, Wu states, at page 8645, left col., that:

> Of the positions identified as being on the surface of the capsid, we found six that potentially are capable of accepting foreign epitope or ligand insertions for

> retargeting the viral capsid to alternative receptors. These are...the loop I region (aa 266), the loop IV region (near aa 447 and 591)...

These positions correspond to the insertion sites, SEQ ID NO: 2 (amino acids 257-266), SEQ ID NO: 4 (amino acids 443-452), and SEQ ID NO: 7 (amino acids 583-592) of Applicants' claims. (The remaining four sites specified by Applicants were not tested by Wu, as indicated by Table 1).

Further evidence of the workability of Applicants' recited AAV insertion sites is provided by Shi and Bartlett (*Molecular Therapy* 7:515, 2003; copy enclosed). This reference states that "insertions following amino acids 139, 584, or 588 were well tolerated and did not affect titer appreciably." Again, insertions at amino acid 584 and 588 are encompassed by SEQ ID NO: 7.

In view of the above evidence, Applicants request withdrawal of the rejections under 35 U.S.C. § 112, first paragraph. Applicants have provided scientific evidence that a number of different insertions can be made in the AAV capsid sequence at the sites particularly specified in Applicants' claims. In particular, of the seven recited positions, Applicants or others have indicated that retargeting insertions can be made at three of these sites, demonstrating not only that these particular sites are useful for retargeting AAV but also confirming the general workability of Applicants' approach to identifying capsid positions amenable to insertions and useful for redirecting viral infectivity. This evidence is nowhere contradicted by reasoning or scientific publications provided by the Office. Applicants' current scope of protection is justified, and the rejections under 35 U.S.C. § 112, first paragraph should be withdrawn.

Rejection under 35 U.S.C. § 102

Claims 1, 29, 31, 32, 35-40, 42, and 43 stand rejected under 35 U.S.C. § 102(a) as being anticipated by Mamounas. As applied to the current claims, this rejection is respectfully traversed.

The rejection is based on the statement that the "broadest interpretation of insertion sites that are before and/or after the recited SEQ ID NO:s include locations that are distinct from the recited amino acids." By the present amendment, Applicants have introduced clarifying claim language consistent with the teaching of the present specification, requiring that the insertion be located directly adjacent to the recited amino acids. As the Office agrees that these particular insertion sites are nowhere disclosed by Mamounas, this basis for the rejection may be withdrawn.

In addition, Applicants wish to correct the record regarding previous arguments made in connection with the Mamounas reference. In Applicants' Reply mailed November 18, 2004, Applicants stated that the Mamounas reference failed to teach a structural protein of AAV that was capable of particle formation. Applicants now believe that statement to be in error in view of Table 2 of Mamounas, disclosing NCI 187 cells transduced with AAV/Ad vp1 hydro SCF, an AAV vector with an SCF insert, that apparently produces virus. Applicants note that this argument is unnecessary for supporting the patentability of the present claims.

Obviousness-Type Double Patenting

Claims 1, 29-32, 35-40, and 42 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 121, 122, 130, and 143 of co-pending Application No. 10/498,163. Applicants wish to defer response to this rejection until such time as allowable subject matter in the '163 application and otherwise allowable subject matter in the present case have been indicated.

Information Disclosure Statement

Applicants draw the Examiner's attention to the Information Disclosure Statement filed in connection with this case on January 31, 2006. Applicants request that the references listed on that Statement be reviewed by the Examiner and an initialed Form

PTO-1449 returned with the next Action from the Office.

CONCLUSION

Applicants submit that the claims are now in condition for allowance, and such action is respectfully requested. Enclosed is a petition to extend the period for replying for one month, to and including April 6, 2006. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 06 April 2006

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RGD Inclusion in VP3 Provides Adeno-Associated Virus Type 2 (AAV2)-Based Vectors with a Heparan Sulfate-Independent Cell Entry Mechanism

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Recombinant adeno-associated virus (AAV) has become an attractive vector system for a number of gene therapy paradigms. However, the utility of AAV vectors is often limited by the absence of heparan sulfate proteoglycan (HSPG), the virus's primary attachment receptor, on the desired target cell population. In order to achieve HSPG-independent gene delivery, several groups have shown that the endogenous tropism of AAV can be expand by genetically altering the viral capsid. However, the parameters of this developing technology have yet to be defined and it has not yet been determined if these modified vectors actually infect cells via these engineered interactions. Previously we constructed a series of insertion mutants spanning the AAV capsid protein gene and identified specific sites that can tolerate the insertion of small exogenous peptides. Here we describe a number of sites within the AAV capsid gene that can be used for the insertion of integrin-targeting peptide epitopes. Incorporation of an Arg-Gly-Asp (RGD)-containing peptide at these sites enables AAV to infect integrin-expressing cells independent of HSPG. Mutant AAV vectors displaying these peptide ligands can be produced to wild-type titer and have been shown to specifically interact with the targeted integrin receptors and mediate infection via this interaction. We report significant increases in gene transfer to Raji, K562, and SKOV-3 cell lines that express integrin, but little HSPG, suggesting that rAAV vectors displaying RGD peptides may be of great utility for treatment of neoplasms characterized by the deficiency of HSPG expression. We have also demonstrated that due to their expanded tropism, these novel vectors are capable of efficient transduction of AAV2-resistant tumors *in vivo* suggesting that they may offer significant therapeutic advantages.

Key Words: AAV, vector targeting, RGD peptide, integrin, gene therapy

Introduction

Gene transfer vectors based on adeno-associated virus type-2 (AAV2) have shown great promise for human gene therapy due to the stability of AAV2-mediated gene expression, lack of significant toxicity or immune response, and the efficiency of gene transfer *in vivo*. However, while AAV2 vectors can effectively transfer genes to a number of different cell types, including muscle, brain, and liver [1], there appear to be conditions which limit transduction of other cell types [2–6]. One such condition is related to the requirement for heparan sulfate proteoglycan (HSPG) [3,7], the primary attachment receptor for AAV2-based vectors, in order to achieve significant levels of gene transfer. In this regard, targeting virus to alternative cell-surface receptors may significantly improve the utility of the present generation of AAV2 vectors for gene therapy.

The initial steps of AAV infection require interaction of specific protein components of the virus capsid with HSPG [7]. This is followed by the internalization of the virus within a clathrin-coated endosome [3] which is thought to be mediated by the interaction of another region of the AAV capsid with $\alpha_v\beta_5$ integrin [8]. Once internalized, the virus escapes from the endosome by triggering its acidification, translocates to the nuclear pore complex, and enters the cell nucleus where subsequent steps of viral uncoating and replication take place [3].

As the capsid protein is the sole mediator of cell entry

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and intracellular trafficking, targeting of recombinant AAV vectors to alternative cellular receptors can be achieved by genetic modification of the capsid [9–13]. We have recently determined that a number of regions within the AAV capsid protein can be altered by the incorporation of small peptides. It has then been hypothesized that if these peptides possess receptor-binding specificities, the virus should be able to attach to and infect cells via these novel interactions [9]. Previously, we have re-directed vector tropism to cells expressing luteinizing hormone receptors by incorporating a small peptide epitope derived from luteinizing hormone into the AAV capsid [9]. Similarly, other groups have investigated the targeting of AAV-based vectors to cell-surface integrin receptors [11,12], serpin receptors [10], and endothelial cell-specific receptors [13]. In each of these cases, AAV-mediated gene transduction was either enhanced or the tropism of the vector was expanded to a previously non-permissive cell type. These accomplishments represent a significant advancement in the filed of AAV-mediated gene transfer. However there remain a number of issues that must be resolved before this technology can be embraced for widespread gene transfer applications.

Importantly, it has not been convincingly demonstrated that infection actually proceeds via the engineered interaction, completely independent of the virus's endogenous receptor. In fact, in one instance transduction of targeted cells with a modified AAV2 vector was inhibited by competition with soluble heparin sulfate [10]. In another instance, gene transduction was maintained in the presence of heparin sulfate, but the role played by the targeted receptor in mediating infection was not determined [13]. The ability to convincingly "re-target" AAV vectors to alternative cellular attachment receptors and alternative pathways of cellular entry has yet to be established. The second issue that must be resolved is a better understanding of the optimum sites within the AAV capsid for insertion of targeting peptide ligands. In each of the previous reports only a small number of sites were investigated. Furthermore, the use of different peptide insertions in these different sites has made if difficult to determine which sites might be best for any one particular peptide ligand.

Previously, we investigated 25 unique sites within the AAV2 capsid protein and determined which could be altered by peptide insertion without significantly affecting vector titer [9]. Capitalizing on this previous work, and the published reports of other groups [10,11], we have compared the best of these sites for their ability to support efficient assembly and packaging of recombinant AAV genomes and for display of a prototypical targeting peptide on the surface of the virus particles. We have chosen the RGD motif as a targeting peptide due to its proven *in vivo* targeting capabilities [14–16] and report the construction of 14 different AAV vectors comprised of capsids containing RGD peptide insertions at 7 different sites within the capsid protein monomers. Furthermore, we show that incorporation of this motif into the AAV VP3 monomer allowed the virus to specifically use the RGD-integrin interaction as an alternative infection pathway, thereby dramatically improving the ability of the virus to transduce several cell types, which are normally poorly infected by this virus. Importantly we show that RGD-modified AAV2 vectors physically interact with the targeted integrin receptors, both in solid-phase binding assays and on the cell surface, and that this interaction is sufficient and required for targeted gene transduction.

Finally, in order to provide a practical means of overcoming limitations imparted by the endogenous viral tropism, targeted AAV vectors must be able to be produced to comparable levels as unmodified vector. In earlier reports there have been large discrepancies in the ability to maintain wild-type titer in targeted AAV vector preparations. Here we show that in addition to promoting efficient display of targeting peptide ligands, the identified sites also allow the maintenance wild-type titer and vector production capabilities.

Results

Generation of AAV Capsid Mutants

It has previously been shown that AAV can tolerate the insertion of exogenous peptide sequences into each of the 3 viral capsid proteins [9]. Importantly, it has been shown that the inserted peptides can be displayed on the surface of AAV particles and can promote the interaction of these viral particles with alternate cell surface receptors [9,11]. To utilize these findings for the purpose of retargeting AAV infection, we introduced a 4C-RGD peptide, CDCRGDCFC, which is known to bind with high affinities to several integrins present on the surfaces of mammalian cells, into each of the AAV capsid protein monomers. This effort was undertaken in an attempt to generate an AAV vector that would bind to cells utilizing a capsid-RGD-integrin interaction as has previously been reported by Girod *et al.* [11] and allow us to define the important parameters of this technology. Based on our previous work [9] and the published work of others [10–12], we selected seven sites within the AAV capsid gene into which to insert RGD-encoding oligonucleotides (Table 1). One site was within the VP1 unique region of the AAV2 capsid protein gene, 3 were within the VP1/VP2 unique region, and the remaining 3 sites were located within the VP3 region of the capsid ORF (Table 1). The mutants were constructed in the non-infectious AAV plasmid, pACG2, by PCR-based site-directed mutagenesis and all were created so that they contained a restriction site at the location of insertion to facilitate confirmation of the mutant sequence and subsequent insertion or swapping of foreign epitopes. A total of 14 different mutants were generated. The 4C-RGD peptide epitope was either inserted alone, or flanked by one of two different 5 amino acid connecting

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TABLE 1: Characteristics of mutant AAV vectors

Virus	Upstream linker	Inserted peptide epitope (4C-RGD)	Downstream linker	Particle titer (ELISA)
A46-RGD4C	TG	CDCRGDCFC	—	8.5×10^7
A46-RGD4CGLS	TG	CDCRGDCFC	GLS	4.5×10^6
A115-RGD4C	TG	CDCRGDCFC	—	4.5×10^6
A115-RGD4CGLS	TG	CDCRGDCFC	GLS	6.0×10^7
A139-RGD4C	TG	CDCRGDCFC	—	8.5×10^7
A139-RGD4CGLS	TG	CDCRGDCFC	GLS	9.0×10^7
A161-RGD4C	TG	CDCRGDCFC	—	4.5×10^6
A161-RGD4CALS	TG	CDCRGDCFC	ALS	5.0×10^6
A459-RGD4C	TG	CDCRGDCFC	—	4.5×10^6
A459-RGD4CGLS	TG	CDCRGDCFC	GLS	4.5×10^6
A584-RGD4C	TG	CDCRGDCFC	—	8.5×10^7
A584-RGD4CALS	TG	CDCRGDCFC	ALS	9.0×10^7
A588-RGD4C	TG	CDCRGDCFC	—	9.0×10^7
A588-RGD4CGLS	TG	CDCRGDCFC	GLS	9.0×10^7
Wild-type	—	—	—	7.5×10^7

peptide linkers [9], at each of the 7 different positions within the AAV capsid protein (Table 1).

These additional sequences were included in an attempt to maintain flexibility and promote efficient display of the engineered RGD epitope on the surface of the AAV particles. We have previously shown that incorporation of these sequences can significantly enhance particle assembly and infectious titer [9].

All of the mutant capsid proteins were able to effectively assemble and package AAV vector genomes (Table 1). Furthermore, all of the resulting AAV vectors were infectious, although there were significant differences in the efficiencies of the different mutant AAV capsids to mediate gene transduction (Figure 1). These differences were related to both the site of peptide insertion and the presence, or absence, of linker sequences flanking the inserted 4C-RGD peptide. Insertion of the RGD epitope following AAV VP1 amino acid 46, 115, 161, or 459 severely diminished infectious titer. However, insertions following amino acids 139, 584, or 588 were well tolerated and did not affect titer appreciably. In all cases, inclusion of linker/scaffolding sequences resulted in

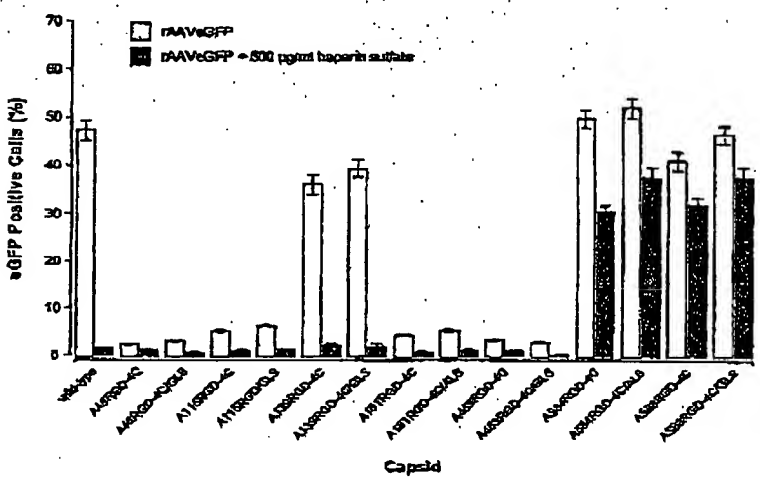

FIG. 1. Gene transduction mediated by mutant AAV vectors. AAV vectors containing 4C-RGD epitope insertions at various sites within the capsid protein were assayed for their ability to direct eGFP gene transduction in the presence and absence of heparin sulfate. Adenovirus-infected HeLa C12 cells (3 iu/cell) were exposed for 2 h at 4°C to RGD-mutant rAAVeGFP vectors or standard AAVeGFP vector (wild-type) at an MOI of 100 pp/cell. Unbound virus was then removed, fresh medium was added, and the cells were analyzed by FACS for eGFP expression after 48 h. Where indicated, the viruses were bound to the cells in the presence of 500 pg/ml heparin sulfate. Data represents the percent of the cell population that expressed the eGFP transgene and is shown as the mean of triplicate samples. Bars indicate the standard error of the mean (SEM).

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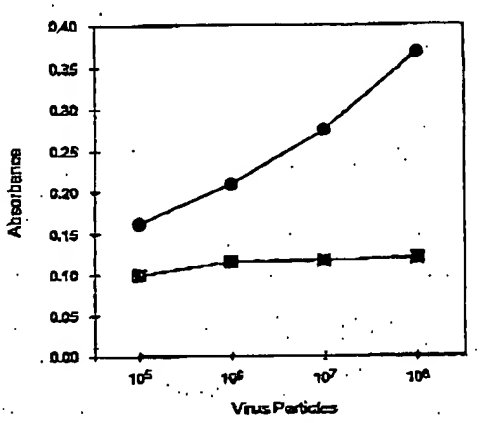

FIG. 2. Solid-phase binding of RGD mutant AAV vector to purified $\alpha_v\beta_3$ integrin. Iodixanol purified AAV vector with either wild-type capsid or A5884C-RGD capsid was bound to assay plates via immobilized heparin sulfate and then incubated with affinity-purified $\alpha_v\beta_3$ integrin. Integrin bound to AAV was detected with anti-α subunit monoclonal antibody, VNR139. ●, A5884C-RGD capsid; ■, wild-type capsid. Assay was performed in triplicate.

slightly more efficient infection and maintenance of titer (Figure 1). We assume that the increases we observed in infection were a direct result of enhanced particle stability, as exemplified by the increased particle titer (Table 1), and supported by our previous work [9]. In an attempt to determine if the inserted 4C-RGD motif had provided the mutant vectors with a HSPG-independent cell entry mechanism, gene transduction assays were also performed in the presence of soluble heparin sulfate [9]. Whereas vectors containing unmodified capsids were unable to transduce cells in the presence of heparin sulfate, mutants containing the 4C-RGD epitope following amino acids 584 or 588 were still able to transduce cells in the presence of heparin sulfate. These results strongly suggested that the mutant vector particles might be directing infection via an alternative cellular receptor.

AAV-RGD Particles Efficiently Interact with Integrins via the RGD Tripeptide

In order to assess the ability of the AAV-RGD particles to bind integrin receptors we employed a solid-phase ELISA assay using purified $\alpha_v\beta_3$ integrin. This analysis clearly showed efficient binding of integrin to immobilized particles of A5884C-RGD AAV, while binding of $\alpha_v\beta_3$ integrin to a control virus was at the background level at all concentrations of virus used (Figure 2). Based on these results, we hypothesized that A5884C-RGD AAV particles are able to interact with other RGD-binding integrins and that this interaction might be responsible for the HSPG-independent gene transfer we observed (Figure 1).

Mutant AAV Vectors Containing RGD Peptide Insertions Have an Expanded Cellular Tropism

Our next goal was to examine whether introduction of the RGD motif in the AAV capsid protein of AAVeGFP resulted in any changes with respect to the ability of the virus to infect different cell types. In order to investigate the entry pathway utilized by A5884C-RGD vectors, we assessed gene transfer to cell lines expressing various levels of HSPG as well as integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The panel of cell lines we used included human HeLa cells; K562, human chronic myelogenous leukemia cells; Raji, human lymphoblast-like cells; and SKOV-3, human ovarian adenocarcinoma cells. While HeLa cells readily support AAV infection, K562, Raji, and SKOV-3 cells are poorly transduced by rAAV vectors [2,22]. Our flow cytometry assay showed that HeLa cells express high levels of HSPG and $\alpha_v\beta_5$ integrins, whereas $\alpha_v\beta_3$ integrins are poorly expressed (Figure 3). K562 cells demonstrated low levels of HSPG expression, no expression of $\alpha_v\beta_3$ integrins, but high expression of $\alpha_v\beta_5$ integrins (Figure 3). Whereas, Raji and SKOV-3 cells expressed little to no HSPG, but expressed high levels of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Therefore, for our subsequent gene transfer experiments we established a set of cell lines covering a range of HSPG expression profiles and with moderate to high levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins present on their cell membranes.

Although K562, Raji, and SKOV-3 cells were poorly transduced by AAVeGFP vectors containing wild-type AAV capsid protein, they were efficiently transduced by the same vector packaged into A5884C-RGD capsids (Figure 4). The efficiency of eGFP gene transfer in these cells mediated by the A5884C-RGD capsid approached that observed in the HeLa cells mediated by the wild-type capsid (Figure 4). Furthermore, our experiments revealed striking differences between the transduction profiles demonstrated by these two viruses (Figure 4). Gene transfer mediated by the RGD-containing particles was 40-fold higher on the K562 cells, 13-fold higher on the Raji cells, and 6-fold higher on the SKOV-3 cells, than gene transfer mediated by particles with wild-type capsids. These experiments clearly showed that incorporation of the 4C-RGD epitope into the VP3 monomer of AAV2 vectors resulted in dramatic changes in the initial steps of virus-cell interaction, presumably by creating an alternative cell attachment and entry pathway.

AAV-RGD Vectors Demonstrate Increased Efficiencies of Cell Binding Due to Utilization of RGD-Integrin Interaction

Having established that A5884C-RGD capsids and wild-type AAV capsids demonstrate different efficiencies of gene delivery as well as different profiles of HS-mediated inhibition of transduction, our next task was to compare the cell binding profiles of these two viruses. To address this issue, we blocked virus binding to HSPG with soluble heparin sulfate, and then detected surface-bound AAV

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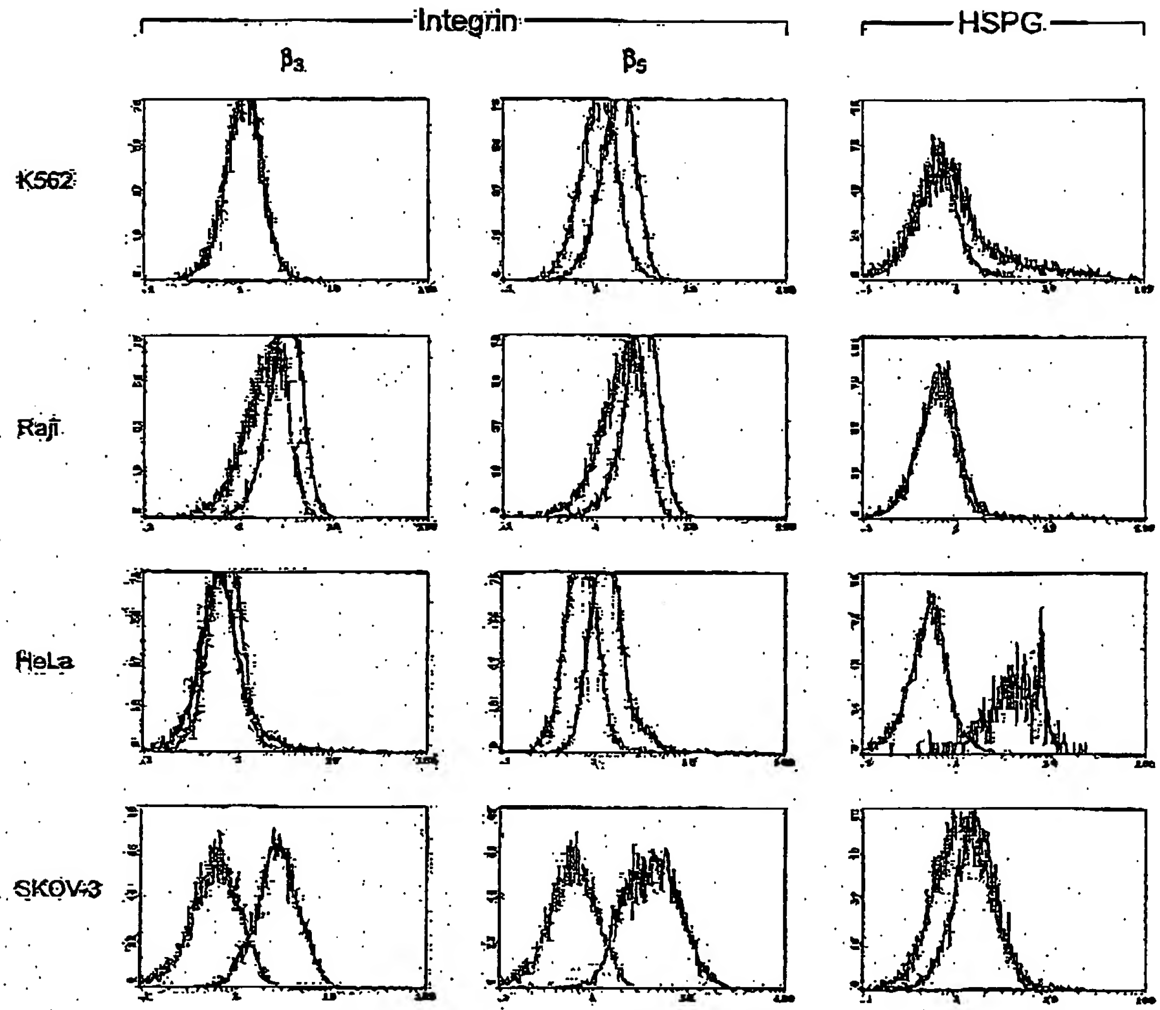

FIG. 3. Integrin and heparan sulfate proteoglycan (HSPG) expression on K562, Raji, HeLa, and SKOV-3 cell lines. Expression of $\alpha_v\beta_3$ integrin was determined using LM609 antibody, whereas $\alpha_v\beta_5$ expression was determined using P1F6 antibody. HSPG expression was determined using HepSS-1 monoclonal antibody. In all cases, secondary FITC-labeled anti-mouse antibody was used and detected by FACS analysis (dark line). The light line shows the results for negative controls. Representative histograms are shown.

using the anti-AAV mAb, A20, and FACS analysis. As shown in Figure 5, AAV comprised of wild-type capsid monomers is not able to bind HeLa cells in the presence of heparin sulfate. However, particles containing A5884C-RGD capsid proteins were still able to bind HeLa cells in the presence of heparin, and it was not until these cells were also treated with synthetic RGD peptide that binding could be completely inhibited. Since the RGD peptides could efficiently block binding, these data further suggest that A5884C-RGD capsids are able to use cellular integrins as alternative receptors during the cell attachment process.

AAV-RGD Vectors are Capable of Mediating Gene Delivery via Integrin Receptors

Having established that A5884C-RGD vectors were able to specifically bind integrins in both solid phase and cell-binding assays, we next utilized these vectors for an assay based on competitive inhibition of AAV-mediated gene delivery by soluble heparin sulfate, known to efficiently block virus binding to cell surface HSPG. Furthermore, we utilized a synthetic RGD peptide and anti-integrin antibody to see if they could also inhibit infection in the presence of soluble heparin sulfate. As shown in Figure 6, eGFP expression in HeLa cells mediated by control virus,

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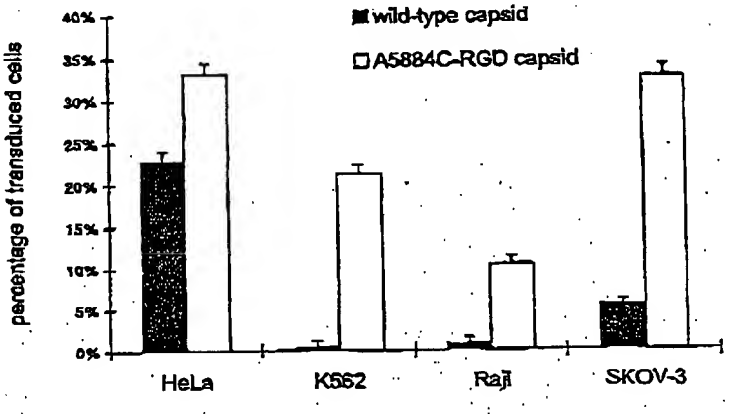
FIG. 4. Gene transduction mediated by A5884C-RGD mutant AAV vector. Vectors displaying the 4C-RGD motif have an increased ability to mediate gene transfer to K562, Raji, and SKOV-3 cells. Cells were exposed for 2 h at 4°C to either RGD-mutant (AAV-A5884C-RGD-eGFP) or standard AAVeGFP vector (wild-type capsid) at an MOI of 100 pp/cell. Unbound virus was then removed, fresh medium was added, and the cells were analyzed by FACS after 48 h. Data represents the percent of the cell population that expressed the eGFP transgene and is shown as the means and standard deviations of triplicate experiments.



AAVeGFP with a wild-type capsid, was efficiently blocked by soluble heparin sulfate. In marked contrast, the same concentration of heparin sulfate was able to block only about 20% of A5884C-RGD capsid-mediated eGFP expression in HeLa cells, thereby indicating that in addition to the well-characterized capsid-HSPG interaction utilized

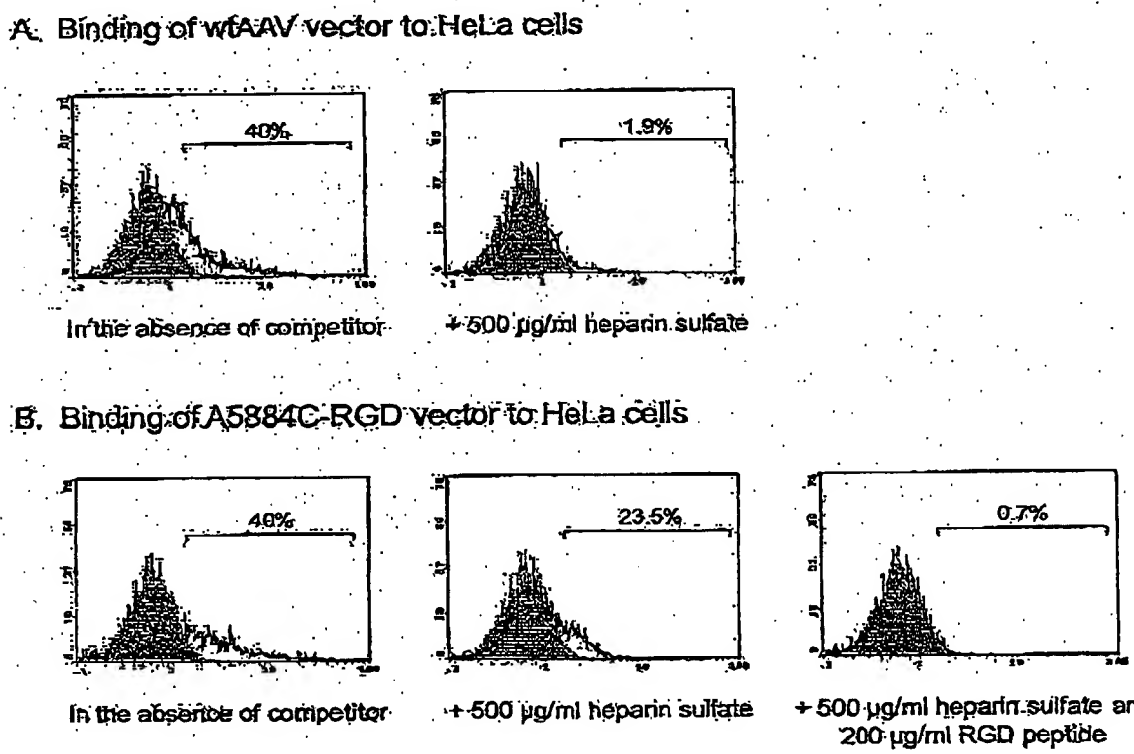


FIG. 5. HSPG-independent binding of A5884C-RGD vector to HeLa cells is mediated by the inserted 4C-RGD motif. Vector particles bound to HeLa cells were detected by staining with anti-AAV A20 mAb and FACS analysis. Histograms from representative experiments are shown. (A) Binding of wild-type AAV (wtAAV) particles to the cell-surface is inhibited in the presence of soluble heparin sulfate (500 µg/ml). Whereas, (B) binding of A5884C-RGD particles is only partially inhibited in the presence of heparin sulfate and not completely inhibited until both heparin sulfate and RGD peptide (200 g/ml) are used. The percentage of cells with bound AAV particles is indicated above the histograms.

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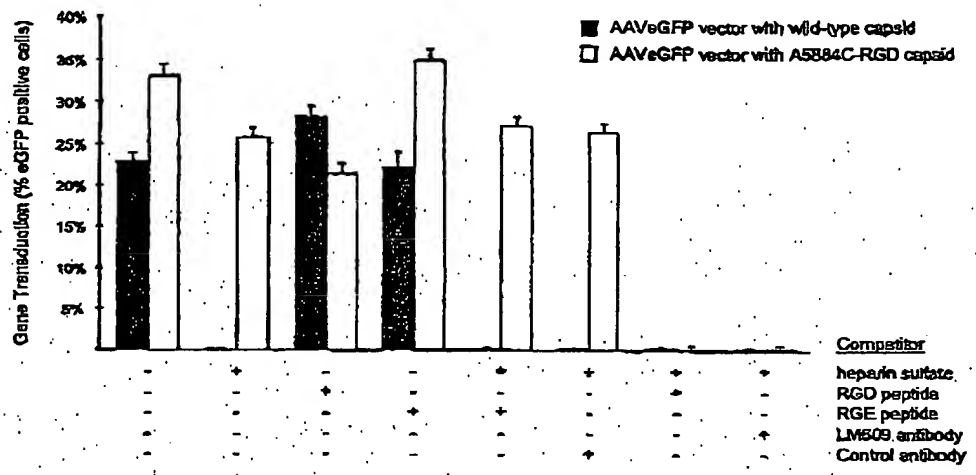

FIG. 6. RGD mutant vectors can use a HS-independent pathway for infection of HeLa cells and are specifically targeted to integrin receptors. HeLa cells were exposed for 2 h at 4°C to either RGD-mutant (AAV-A5884C-RGD-eGFP) or standard AAVeGFP vector (wild-type capsid) at an MOI of 100 pp/cell. Unbound virus was then removed, fresh medium was added, and the cells were analyzed for eGFP gene transduction by FACS after 48 h. For the competition experiments, the viruses were bound to the cells for 2 h at 4°C in the presence of 500 μg/ml heparin sulfate, 200 μg/ml RGD-peptide, 200 μg/ml RGE-peptide, and/or 1:200 dilution of anti-integrin or isotype-matched control antibody. Data represents the means and standard deviations of triplicate experiments.

by wild-type AAV, A5884C-RGD capsids are capable of using an alternative, HSPG-independent cell entry pathway. We assume that the partial reduction in gene transduction observed in the presence of HS is due to loss of the HSPG-dependent cell entry pathway. The contribution of the alternative pathway of cell entry was quite significant accounting for entry of approximately 80% of total transducing vector particles. To assess the specificity of this new interaction infections were performed in the presence of either excess free RGD peptide or anti-integrin antibody in conjunction with soluble heparin sulfate. Both of these competitors were able to completely abolish A5884C-RGD-mediated gene expression when utilized in conjunction with heparin sulfate, while no inhibition was observed with control RGE peptide or isotype-matched control antibody. These data demonstrate the specificity of the engineered interaction for RGD-binding integrins expressed on the cell surface (Figure 6).

Mutant AAV Vectors Containing RGD Peptide Insertions Mediate Enhanced *In Vivo* Gene Delivery to SVOV-3 Adenocarcinomas

Having established that A5884C-RGD vectors were able to target infection via cell-surface integrin receptors and mediate efficient gene transfer to SKOV-3 cells and other cell types that were poorly transduced by unmodified AAV2 vectors, we next utilized these vectors for *in vivo* gene transfer to a murine model of ovarian cancer. As shown in Figure 7, eGFP expression in peritoneal SVOV-3 tumors mediated by control virus, AAV2eGFP with a wild-type capsid, was inefficient when assessed 15 days after vector administration. In marked contrast, the same dose of AAV2eGFP vector with an A5884C-RGD capsid was much more efficient, and transgene product could be detected in essentially all tumor cells by 35 days post vector administration. These experiments clearly show that incorporation of the 4C-RGD epitope into the VP3 monomer of AAV2 vectors results in dramatic changes in the *in vivo* gene transduction potential of these vectors and greatly increases their utility for treatment of neoplasms characterized by the deficiency of HSPG expression.

Discussion

AAV vectors efficiently transduce a wide variety of cells. However, it has recently been established that low levels of HSPG-mediated vector binding limits AAV transduction of several important gene therapy target cells [2,22–24]. It is also likely that other, yet uncharacterized, cell types may likewise lack sufficient levels of HSPG to permit efficient gene transduction. The significant increases in transduction of cells lacking HSPG shown here demonstrates that genetically modified, targeted AAV vectors can be successfully used to expand the range of tissues amenable to efficient AAV-mediated gene therapy.

In this study we describe the generation and characterization of a panel of recombinant AAV vectors containing 4C-RGD epitope insertions within their capsids. These vectors were constructed in an effort to reconcile previous reports of the generation of targeted AAV vectors, identify optimal sites for peptide insertion, define the receptor-

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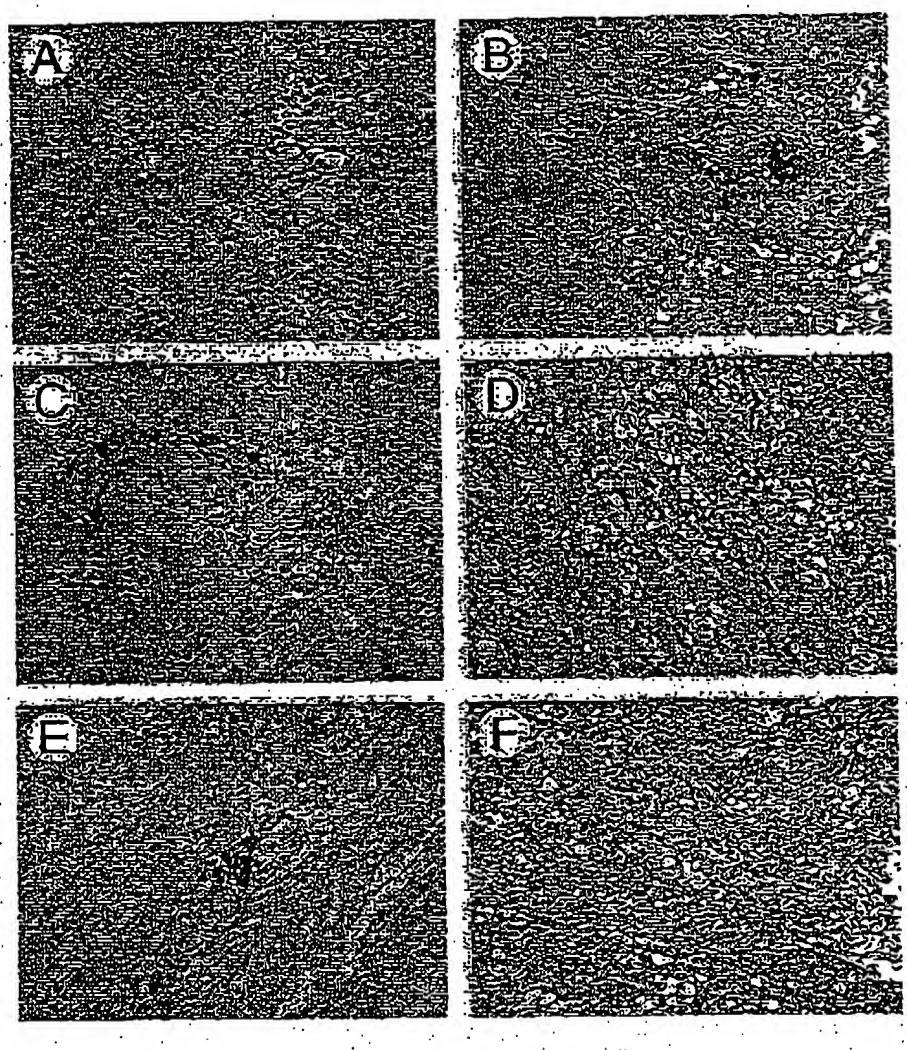

FIG. 7. Gene transfer to an orthotopic murine model of peritoneal ovarian cancer mediated by AS884C-RGD mutant AAV vector. Tumors were established by i.p. injection of 1×10^7 SKOV-3 cells. Five days later 1×10^{10} DRP of either AAV2-eGFP (panels A, C, and E), or AAV-AS884C-RGD-eGFP (panels B, D, and F) was administered i.p. To determine the efficiency of AAV vector-mediated eGFP expression in the tumors, mice were sacrificed on days 15 (A and B), 25 (C and D) and 40 (E and F) after vector administration. Tumor nodules were dissected and immunostaining for eGFP was performed on 4-μm paraffin sections counterstained with hematoxylin. Vectors displaying the 4C-RGD motif were considerably more efficient in mediating gene transfer to these tumors *in vivo*.

binding specificities of these vectors, and further demonstrate the utility of peptide insertions for targeting AAV vectors to ligand-specific receptors. We took advantage of the well-characterized interaction between cellular integrins and various proteins containing RGD tripeptides. This interaction plays an important role in a variety of fundamental biological processes, including cell adhesion and viral infection. Furthermore, integrin expression is activated in a wide variety of tumors [25–29] and targeting AAV to these receptors might allow the specific delivery of a gene while avoiding delivery to surrounding healthy tissue. Girod *et al.* [11] reported that genetic incorporation of RGD-containing sequences into AAV2 capsid protein expanded tropism to previously non-permissive cell types. Similarly, recent studies have shown that incorporation of RGD-peptides into human adenovirus fiber proteins permits specific interaction of these viral particles with cellular integrins and infection of target cells via this interaction [14,16].

We constructed 14 different RGD-capsid insertion mutants and showed that these mutant capsid proteins could still assemble into AAV particles and efficiently package recombinant AAV vector genomes. The crystal structure of AAV2 has recently been reported [30]. The core structure is a β-barrel motif, consisting of anti-parallel β-sheets with interspersed looped-out domains. The largest of these loops is located between β-sheets G and H (GH loop) and contains the majority of sites that have been shown to be amenable to manipulation [9]. The optimum sites for epitope insertion appear to define a subdomain within this loop on the basis of surface exposure and local structural flexibility. By using an ELISA-based binding assay,

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we were then able to show direct interaction of RGD-modified viral particles with purified integrin $\alpha_v\beta_3$. Furthermore, we were able to show that these modified AAV particles were able to bind integrin receptors on the surface of appropriate target cells and utilize this interaction for cell entry, thereby supporting the concept of augmented efficiency of transgene expression as a result of more-efficient primary interaction between the virus and the target cell and specific targeting via an alternative cellular receptor.

The modified RGD-containing AAV vectors display a significantly different profile of gene transfer compared to AAV2 vectors with unmodified capsid. This difference was especially dramatic when HSPG-negative cell lines were utilized for gene delivery experiments. Targeting gene delivery via the RGD-integrin interaction also demonstrates that attachment via HSPG is not required for efficient transduction of cells by AAV. A previous attempt to target AAV vectors via peptic insertion of a serpin receptor ligand showed a continued dependence on HSPG [10], which was not observed in this study. Transduction of receptor-blocked HeLa cells using soluble heparin sulfate and RGD-targeted AAV vector was nearly as efficient as transduction with un-modified vector in the absence of competitor. Therefore, in the absence of HSPG-mediated binding, the transducing activity of an AAV particle is not compromised by attachment via an alternative receptor. Although several cell types may lack HSPG expression, a number of tissues are known to express the AAV receptor. Therefore, the efficient targeting and restriction of transduction to a particular cell type or tissue will necessitate AAV vectors that lack HSPG-binding activity. It will be important to determine the levels of HSPG expression in tissues targeted for AAV-mediated gene therapy. If the levels of HSPG expression are low, using AAV vectors that have been engineered to interact with an alternative receptor is likely to increase the efficiency and specificity of gene transfer.

Finally, we have used an RGD-targeted AAV vector to assess gene transfer to a stringent orthotopic murine model of peritoneal ovarian cancer and show significant improvement in gene transfer efficiency. These results suggest that AAV vectors packaged into capsids containing VP3 4C-RGD epitope insertions could be effective agents for the treatment of ovarian cancer and other cancers characterized by low HSPG and moderate RGD-binding integrin expression.

Materials and Methods

Cells and tissues. Low passage number (passage number 20–40) HEK 293 cells [17], HeLa cells, and HeLa C12 cells [18] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml), at 37°C and 5% CO_2. Raji, a human lymphoblast-like cell line, and K562, a human chronic myelogenous leukemia cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 containing 10% FBS. SKOV-3, a human adenocarcinoma cell line, was also obtained from ATCC and grown in DMEM containing 10% FBS.

Enzymes. Restriction endonucleases, T4 DNA ligase, Platinum *pfx* DNA polymerase, benzonase and proteinase K were purchased from Life Technologies (Gaithersburg, MD).

Monoclonal antibodies. Anti-$\alpha_v\beta_3$ integrin monoclonal antibody 1M609 and anti-$\alpha_v\beta_5$ integrin monoclonal antibody P1F6 were purchased from Chemicon International, Inc. (Temecula, Calif). Anti-bacterial β-galactosidase monoclonal antibody was used as an isotype-matched (IgG_1) control and was also purchased from Chemicon. Anti-human α_v integrin monoclonal antibody VNR139 was purchased from Life Technologies (Gaithersburg, MD). Anti-heparan sulfate, HepSS-1, purified mouse IgM antibody was purchased from Seikagaku America (Falmouth, MA) and FITC-labeled secondary goat anti-mouse IgG (H + L) antibody was obtained from either Chemicon or Vector Laboratories (Burlingame, CA). Anti-AAV2 monoclonal antibody A20 was obtained from American Research Products, Inc. (Belmont, MA).

ELISA. Solid-phase binding assays were performed by the following method. Neutravidin-coated plates (Pierce) were incubated with 1 μg/well of biotinylated heparin in PBST (0.05% Tween 20, 0.2% bovine serum albumin (BSA)) overnight at 4°C. The wells were then washed five times with wash buffer (PBS containing 0.05% Tween-20 and 0.1% BSA) and AAV particles were bound at room temperature (RT) for 2 h with gentle shaking. The plate was then washed five times with wash buffer and purified integrin $\alpha_v\beta_3$ (Chemicon) in binding buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1% BSA, pH 7.5) was added to each well at a concentration of 1 μg/ml. The plates were incubated over night at 4°C, washed three times with wash buffer and incubated with VNR139 mAb (GIBCO-BRL; anti-α_v subunit, 1:2000 dilution) in binding buffer for 2 h at RT. The plates were then washed five times and incubated with secondary antibody (HRP-conjugated anti-mouse IgG) for 1 h at RT. Following a final wash the ELISA plate was developed with ABTS substrate solution and VECTASTAIN kit (Vector Laboratories) as recommended by the manufacturer. Color development was stopped by the addition of 1N H_2SO_4, and plates were read in a plate reader set at 405 nm.

Construction of recombinant AAV packaging plasmids. Recombinant AAV constructs encoding capsid proteins with inserted 4C-RGD peptide epitopes were generated as described previously [9]. Briefly, DNA primers were designed encoding the peptide sequence and used to direct PCR-based mutagenesis of plasmid pACG2 [19]. This plasmid contains the AAV genome, less the two viral ITRs, and an ATG-to-ACG mutation of the *Rep78/68* start codon which has been shown to increase rAAV vector yield by attenuating Rep78/68 synthesis [20]. In some instances additional non-homologous flanking sequences were included in the PCR primers. These sequences encoded linker/scaffolding sequences on either side of the RGD epitope insertion. The PCR products were digested with *DpnI* endonuclease to eliminate the parental plasmid template and were propagated in DH-5α bacteria (Life Technologies). Mini-prep DNAs were extracted from ampicillin resistant colonies and were screened by restriction endonuclease digestion.

Viruses. Recombinant AAV vector, AAVeGFP, containing the enhanced green fluorescent protein gene (eGFP) driven by the human CMV IE promoter/enhancer region packaged into wild-type AAV2 capsids was produced by triple-transfection as previously described [9]. To produce rAAV with RGD-containing capsid proteins, 293 cells were transfected with mutant pACG2 plasmids, constructed as described above, in place of normal pACG2 plasmid. Transfections were carried out at 37°C using the calcium phosphate transfection system (Life Technologies) according to the manufacturer's specifications. Forty eight hours after transfection, cells were harvested by centrifugation at 500 × g for 10 min, resuspended in PBS, and recombinant virus was released by freezing and thawing three times. The crude lysate was clarified by centrifugation at 500 × g for 10 min and treated with benzonase at 250 U/ml final concentration at 37°C

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for 30 min. Virus was further purified by iodixanol step gradient and heparin sulfate affinity chromatography [21] and stored at −20°C in PBS containing 20% glycerol. Particle titers were determined by ELISA and DNA dot blot as previously described [9], and infectious titers were determined by gene transduction assay on HeLa C12 cells in the presence of adenovirus type-5 (Ad5) at 2 hu/cell as described below.

Flow cytometry. For analysis of integrin expression, adherent cells were released from culture flasks by the addition of EDTA and resuspended in SM buffer (HEPES-buffered saline containing 1% BSA) at 2×10^6 cell/ml as described by Dmitriev *et al.* [16]. Cells were incubated briefly at 37°C to allow regeneration of surface integrin, then incubated with FITC-labeled LM609 antibody, or FITC-labeled PIF6 antibody (1:200 dilution) for 2 h at 4°C, washed five times with SM buffer and analyzed by flow cytometry at the Children's Research Institute Cell Morphology Core Facility. For analysis of HSPG expression, 2×10^6 cells were incubated with anti-HSPG monoclonal antibody, HepSS-1, at a dilution of 1:200 for 2 h at 4°C. The cells were then washed five times with SM buffer and incubated with FITC-labeled goat anti-mouse IgM serum (1:800) for 1 h at 4°C. The cells were then washed with SM buffer and analyzed by flow cytometry.

Gene transduction assays. Briefly, HeLa C12, Raji, K562, or SKOV-3 cells were seeded in 24-well plates the day before infection so that they would reach about 75% confluence or about 5×10^5 cells/ml the next day. Serial dilutions of wild-type AAVeGFP and RGD-mutant AAVeGFP preps were added to the cells which were then infected with Ad5 at an MOI of 3 hu/cell. The cells and viruses were incubated at 37°C for 48 h, after which time the media was removed and the cells were washed twice with PBS, fixed, and analyzed for GFP gene transduction by FACS. Data has been presented as the percentage of transduced cells in the cell population infected at the indicated particle multiplicity. Transduction assays were also carried out in the absence of adenovirus with no differences in the relative titers of mutants.

For determination of HSPG-independent gene transduction, AAV vectors were first incubated with 1500 μg/ml heparin sulfate for 2 h at 37°C, then incubated with target cells at 4°C in the presence 500 μg/ml heparin sulfate for another 2 h. The cells were then washed three times with fresh medium to remove unbound vector and incubated for 48 h at 37°C prior to determination of gene transduction. For determination of RGD-dependent gene transduction, rAAV vectors were incubated with either RGD-peptide (RGDS, Sigma: 200 μg/ml), RGE-peptide (RGES, Sigma: 200 μg/ml), anti-integrin monoclonal antibody (LM609), or isotype-matched control antibody, in addition to heparin sulfate at 4°C as described above.

Cell-surface binding assays. Cells were resuspended in binding buffer containing 5% FBS at concentration of 2×10^6 per ml. AAV vectors (±heparin sulfate, 500 μg/ml) were added and incubated with the cells for 2 h at 4°C, the cells were then washed three times and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then blocked with 10% horse serum and 0.05% Tween-20 in PBS and incubated with A20 anti-AAV mAb for 4 h at 4°C, and then with FITC-labeled secondary antibody in PBS containing 0.05% Tween-20 and 0.2% BSA for 1h at RT. After a final wash, the cells were analyzed by flow cytometry.

Ovarian carcinoma model, immunostaining and histopathology. CB17 SCID mice (n = 12 mice/group) were injected with 1×10^7 SKOV-3 cells intraperitoneal (i.p.) on day 0. On day 5, mice were injected i.p. with 1×10^{10} DNase-resistant particles (DRP) of AAV2-eGFP, RGD-mutant AAVeGFP (AS884C-RGD AAVeGFP), or no virus. To determine the efficiency of AAV vector-mediated eGFP expression in the tumors, twelve mice (four per group) were sacrificed on days 15, 25 and 40 after vector administration. Immunostaining for eGFP was performed on 4-μm paraffin sections. Following deparaffinization and blocking of endogenous peroxidases, slides were heated for 1 h at 65°C, 190 kpa, in citrate buffer to recover antigen. Sections were then blocked with 10% dry milk and incubated with rabbit anti-GFP antibody (Research Diagnostics, Inc., Flanders, NJ) for 12–18 h in a humidified chamber. An equivalent concentration of rabbit IgG was used as a reagent negative control on matching sections. Biotinylated secondary antibody (Goat anti-rabbit, Vector Laboratories) was applied to the slides for 1 h at room temperature. Sections were incubated with avidin-peroxidase complex (Vector Rabbit Elite ABC kit) following the manufacturer's instructions. Either diaminobenzedine (DAB) or 3-amino-9-ethylcarbazole (AEC) were used as chromagens and counterstained with hematoxylin (Vector Laboratories). Slides were dehydrated with xylene and mounted (Permount) before examination on a Nikon E600 microscope. Representative images were collected using a color video camera.

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